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Phagocytic activities of hemocytes from the deep-sea symbiotic mussels *Bathymodiolus japonicus*, *B. platifrons*, and *B. septemdierum*

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ABSTRACT

Deep-sea mytilid mussels harbor symbiotic bacteria in their gill epithelial cells that are horizontally or environmentally transmitted to the next generation of hosts. To understand the immune defense system in deep-sea symbiotic mussels, we examined the hemocyte populations of the symbiotic *Bathymodiolus* mussel species *Bathymodiolus japonicus*, *Bathymodiolus platifrons*, and *Bathymodiolus septemdierum*, and characterized three types of hemocytes: agranulocytes (AGs), basophilic granulocytes (BGs), and eosinophilic granulocytes (EGs). Of these, the EG cells were the largest (diameter, 8.4–10.0 μm) and had eosinophilic cytoplasm with numerous eosinophilic granules (diameter, 0.8–1.2 μm). Meanwhile, the BGs were of medium size (diameter, 6.7–8.0 μm) and contained small basophilic granules (diameter, 0.3–0.4 μm) in basophilic cytoplasm, and the AGs, the smallest of the hemocytes (diameter, 4.8–6.0 μm), had basophilic cytoplasm lacking granules. A lectin binding assay revealed that concanavalin A bound to all three hemocyte types, while wheat germ agglutinin bound exclusively to EGs and BGs. The total hemocyte population densities within the hemolymph of all three *Bathymodiolus* mussel species were similar ($8.4–13.3 \times 10^5$ cells/mL), and the percentages of circulating AGs, BGs, and EGs in the hemolymph of these organisms were 44.7–48.5%, 14.3–17.6%, and 34.3–41.0%, respectively. To analyze the functional differences between these hemocytes, the phagocytic activity and post-phagocytic phagosome–lysosome fusion events were analyzed in each cell type using a fluorescent Alexa Fluor® 488-conjugated *Escherichia coli* bioparticle and a LysoTracker® lysosomal marker, respectively. While the AGs exhibited no phagocytic activity, both types of granulocytes were phagocytic. Of the three hemocyte types, the EGs exhibited the highest level of phagocytic activity as well as rapid phagosome–lysosome fusion, which occurred within 2 h of incubation. Meanwhile, the BGs showed lower phagocytic activity and lower rates of phagosome–lysosome fusion than the EGs. These findings indicate that the two types of granulocyte play distinct roles in the defense system.

1. Introduction

Deep-sea mussels belonging to the genus *Bathymodiolus* are found in animal communities in or on seeps, hydrothermal vent areas, whale falls, and sunken woods. These animals are known to harbor methane-oxidizing and/or thioautotrophic symbiotic bacteria in their gill epithelial cells [1–4], which supply the host with organic carbon and energy, and are either horizontally or environmentally transmitted to the next generation of hosts [1,4]. While bivalves recognize exogenously invading microorganisms and activate their immune systems to maintain homeostasis [5,6], *Bathymodiolus* mussels accept symbiotic bacteria in their gill epithelial cells.

The family Mytilidae comprises non-symbiotic mussels, such as those belonging to the genus *Mytilus*, and symbiotic mussels, including *Bathymodiolus* species. It was reported that the common ancestor of the symbiotic Mytilidae mussels acquired an extracellular thioautotrophic symbiont, and that intracellular symbiosis and the methanotrophic symbiont appeared later during the course of evolution [7]. *Bathymodiolus* mussels harbor intracellular symbionts [7]. Phylogenetic studies have revealed that *Bathymodiolus*

Abbreviations: AG, agranulocyte; BG, basophilic granulocyte; EG, eosinophilic granulocyte.

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septemdierum and *Bathymodiolus azoricus* are closely related and form a robust clade (Group 2 in Ref. [8]). However, while the former harbor a thioautotrophic symbiont, the latter harbors both thioautotrophic and methanotrophic symbionts [8]. Meanwhile, *Bathymodiolus japonicus* and *Bathymodiolus platifrons* are also closely related (belonging to Group 1-1 in Ref. [8]) and both species harbor a methanotrophic symbiont [3,7,8]. Mytilid mussels might therefore serve as a model bivalve system for studying the evolution of the immune defense system during the evolutionary development of symbiosis.

Hemocytes play critical roles in the immune system of invertebrates. In bivalves, hemocytes are essential for a number of processes, including wound healing, transport and digestion of nutrients, and immune defense [9–13]. Bivalves have two major types of hemocytes: agranular hemocytes, which lack granules, and granular hemocytes, which contain granules [9,12,13]. In mytilid mussels, hemocytes have been classified/characterized using a variety of methods, including density gradient centrifugation [14–16], morphological analysis via light and transmission electron microscopy [14,16–19], and lectin binding assay analyses [19,20]. While three or more types of hemocytes have been reported in bivalves, the nomenclature for these types varies between bivalve species [11]. For example, *B. azoricus* has agranular hemocytes, which are referred to as a hemoblast-like cells, as well as two granular types known as hyalinocytes and granulocytes [19]. Meanwhile, in *Mytilus edulis*, the agranular hemocytes are known as agranulocytes and the two types of granular hemocytes are referred to as basophilic and eosinophilic granulocytes [14]. Finally, *Mytilus galloprovincialis* has agranular hemocytes that are called hyalinocytes, and three types of granular cells referred to as basophilic granulocytes, acidophilic granulocytes, and intermediate cells, which contain both acidophilic and basophilic granules [16].

Phagocytosis is the process by which certain immune cells recognize and eliminate non-self-components, such as invading and/or associating microorganisms, and is one of the most important defensive functions of hemocytes [5,21,22]. In mytilid mussels such as *M. edulis*, *M. galloprovincialis*, and *B. azoricus*, granular hemocytes were reported to exhibit higher phagocytic activities than the agranular type of hemocytes [14,19,23]. However, the phagocytic activity of agranular hemocytes varies depending on the species. For example, while the agranular hemocytes of *M. edulis* were reported to exhibit little phagocytic activity [14], those in *M. galloprovincialis* and *B. azoricus* show moderate activity [18,19,23,24]. After phagocytosis, the phagosome fuses with the lysosome resulting in the intracellular digestion of the ingested microorganisms [22]. Although phagocytosis has been studied in mytilid mussels, the phagosome–lysosome fusion in these organisms has yet to be examined.

To understand the functional differences between the hemocytes of deep-sea symbiotic mussels, it is important to characterize the phagocytic activity of these hemocytes by which they recognize and eliminate non-self-components, such as invading and/or associating microorganisms. In the present study, we therefore aimed to characterize the phagocytic activity and subsequent phagosome–lysosome fusion events in the circulating hemocyte populations of three symbiotic *Bathymodiolus* mussel species.

2. Materials and methods

2.1. Collection of deep-sea symbiotic mussels

Deep-sea *B. japonicus* and *B. platifrons* mussels were collected at a seep off of Hatsushima Island in Sagami Bay, Japan, by the Remotely Operated Vehicle (ROV) Hyper-Dolphin (dive #. 1125, 1128, and 1129; 35°00,929–35°00,973N: 139°13,248–139°13,382E; depth, 817–1237 m) during the R/V Natsushima cruise (NT10-08; May 11–18th, 2010). Water temperature, salinity, and dissolved oxygen were measured with sensors on the ROV and were 4.0–4.5 °C, 34.33–34.35% and 1.1–1.3 mL/L, respectively. *B. septemdierum* mussels were collected at a hydrothermal vent area at the Myojin Knoll, Japan, by the ROV Hyper-dolphin (dive #. 1284 and 1288; 32°6,234–32°6,278N: 139°52,081–139°52,152E; depth, 1228–1303 m) during the R/V Natsushima cruise (NT11-09; June 15–26th, 2011). Water temperature, salinity and dissolved oxygen were 4.6 °C, 34.24‰ and 1.4 mL/L, respectively. The mussels were immediately transferred to the laboratory on the R/V and kept in tanks containing 60 L of filtered (filter pore size, 0.2 μm) seawater (salinity, 3.5%) at 4–5 °C. After the cruises, the mussels were maintained in tanks with artificial seawater (ASW; 3.5% Rohto Marine [Rei-Sea Ltd., Tokyo, Japan] in tap-water) at 4–5 °C in land laboratories until further use. Because the population density of the symbiotic bacteria is known to decrease during cultivation at low sulfide concentrations in the laboratory [25], all animals were used within a week after collection.

2.2. Collection of hemolymph

Hemolymph was withdrawn from the posterior adductor muscle of each mussel using a 5-mL syringe and a 25-gauge needle. The hemolymph (2–10 mL) was transferred to a 2.0- or 15-mL plastic tube, and kept on ice for less than 5 min until further use.

2.3. Harvesting and separation of hemocytes

Hemocytes were harvested from fresh hemolymph (3–5 mL) by centrifugation at 180 × *g* for 5 min at 4 °C, and were fixed in 10 mL of 2.5% glutaraldehyde in filtered ASW (FASW), which was made by filtration of ASW using a Stericap with a filter pore size of 0.2 μm (Millipore Corporation, Billerica, USA), for 2 h at 4 °C. The cells were then harvested by centrifugation (180 × *g*, 5 min at 4 °C), washed with 10 mL of FASW, and concentrated to 1 mL in FASW by centrifugation. The cells were then layered on a 7 mL Percoll (GE Healthcare Ltd., Little Chalfont, UK) density gradient (10–40%) in FASW, prepared using a gradient maker (Sanplatec Corp., Osaka, Japan), and centrifuged at 500 × *g* for 30 min at 4 °C. The separated layers of hemocytes were individually aspirated using a Pasteur pipette, transferred to new 1.5-mL plastic tubes, and washed with 1 mL of FASW. The gravitational densities of the hemocytes from four individual samples of each of the three mussel species were estimated using standard density marker beads (GE Healthcare). The percentages of the hemocyte types in each of the density layers were determined by microscopic observation after staining with May-Grünwald–Giemsa (MGG) stain.

2.4. Light microscopy of hemocytes

After centrifugation of the hemolymph (3–5 mL), the harvested hemocytes were fixed in 10 mL of 4% paraformaldehyde in FASW for 1 h at 4 °C. The cells were then washed twice in FASW at 4 °C and centrifuged at 180 × *g* for 5 min at 4 °C. The collected hemocytes were placed on glass slides (S2215, Matsunami Glass Ind. Ltd., Osaka, Japan) and stained with MGG (first with an undiluted solution of May-Grünwald [Wako Pure Chemical Ind. Ltd., Osaka, Japan] for 1 min and then with Giemsa stain [Muto Pure Chemical Ind. Ltd., Osaka, Japan] diluted 1/10 in 0.1 M phosphate buffer [pH 6.2] at room temperature for 1 min). The cells were then washed with pure water (PW), air-dried, and mounted using a mounting medium (Entellan® Neu, Merck, Darmstadt, Germany) and cover slips. Cells were observed under an Optiphot light microscope (Nikon Co. Ltd., Tokyo, Japan), and cell population densities were

determined using a flat-type disposable hemocytometer (C-chip; NanoEnTek Inc., Seoul, Korea). The total hemocyte population densities and the density of each hemocyte type were determined using seven individual samples from each of the three mussel species. The diameters of the cells and nuclei were determined by measuring approximately 200 cells of each hemocyte type.

Semi-thin sections of hemocytes were generated to observe their granular structures in detail. The cells were fixed by incubating in 4% paraformaldehyde in FASW for 1 h at 4 °C, washed with FASW at 4 °C, and centrifuged at 180 × *g* for 5 min at 4 °C. The cell pellet was embedded in 1.0% agarose in PW, dehydrated using a graded series of ethanol (30%, 50%, 70%, 90%, and 100%), and embedded in Technovit 8100 resin (Heraeus Kulzer Co. Ltd., Hanau, Germany) at 4 °C. Semi-thin sections (thickness, 1 μm) were cut with a diamond knife mounted on an Ultracut S ultra-microtome (Leica Microsystems, Wetzlar, Germany), collected on glass slides (S9445, Matsunami), and stained with MGG. Thin sections were mounted on a glass slide with the mounting medium and observed under a light microscope (Optiphot, Nikon Co. Ltd.).

2.5. Transmission electron microscopy (TEM)

Hemocytes isolated from fresh hemolymph (3–5 mL) of each mussel were fixed with 2 mL 2.5% glutaraldehyde in 0.1 M cacodylate buffer [pH 7.4] containing, 0.3 M NaCl, and 0.15 M sucrose in 15-mL vials, and then immediately centrifuged at 180 × *g* for 5 min at 4 °C. The precipitated cells were re-suspended and re-fixed by incubating in 10 mL of 2.5% glutaraldehyde in the same buffer for 1 h at 4 °C, and then washed in the same buffer at 4 °C, and again centrifuged at 180 × *g* for 5 min at 4 °C. The cell pellets were embedded in 1.0% agarose in the same buffer, and post-fixed by incubating for 1 h at 4 °C in 0.1 M cacodylate buffer (pH 7.4) containing 2.0% osmium tetroxide. After fixing, the cells in the agarose gel were washed with PW, stained by incubating in 1.0% uranyl acetate solution in PW for 1 h at room temperature, dehydrated using a graded series of ethanol (30%, 50%, 70%, 80%, 90%, 95%, and 100%), cleared in n-butyl-glycidyl-ether (Nisshin EM Co., Ltd., Tokyo, Japan), and embedded in Epon 812 (Nisshin EM Co., Ltd., Tokyo, Japan). Ultra-thin sections (thickness, 60 nm) were cut with a diamond knife on an MT2-B Porter-Blum ultra-microtome (Dupont-Sorvall, Newtown, CT, USA), collected on Formvar-coated copper grids, stained with a 2.0% uranyl acetate solution and a 2.0% lead citrate solution, and coated with carbon. The sections were observed using a Tecnai G^2 20 electron microscope (FEI, Hillsboro, OR, USA) operated at 120 kV.

2.6. Lectin binding assay

Four types of Alexa Fluor® 594-conjugated lectins were used for lectin binding assays: wheat germ agglutinin (WGA), concanavalin A (Con-A), soybean agglutinin (SBA), and phytohemagglutinin-L (PHA-L). The hemocytes were fixed in 10 mL of 2.5% glutaraldehyde in FASW for 1 h at 4 °C, washed in FASW at 4 °C, centrifuged at 180 × *g* for 5 min at 4 °C, and embedded in 1.0% agarose in FASW. After the medium was changed from FASW to a graded series of sucrose (10%, 20%, and 30% in FASW; incubation in each for 2–12 h at 4 °C), the cells in the agarose gel were then embedded in 3 mL of Tissue-Tek® O.C.T.™ compound (Sakura Finetek Japan, Co., Ltd., Tokyo, Japan), and frozen in a small volume of hexane floating on liquid nitrogen. The frozen sections (thickness, 4 μm) were cut using a Microm cryostat HM550, collected on a glass slide (S9445, Matsunami), and air-dried for 12 h at room temperature. Sections were then incubated with each of the four fluorescently-labeled lectins diluted in FASW (0.05 mg/mL) for 15 min at room temperature, washed with FASW, and stained with 4′,6-diamidino-2-phenylindole (DAPI; 2 μg/mL in distilled water) for 1–2 min. Stained sections were air-dried for 15 min, mounted with a mounting medium containing a fluorescence quenching inhibitor (Fluoromount™; Sigma–Aldrich, St. Louis, MO, USA) and a cover slip, and then observed under an Eclipse E600 fluorescence microscope (Nikon). The stained nuclei (DAPI) were observed using a UV-1A filter (Excitation wavelength [Ex], 365/10 nm and emission wavelength [Em] > 400 nm), and red fluorescence was observed using a CY3 filter (Ex, 530–570 nm and Em, 573–648 nm).

2.7. Phagocytosis assay

After centrifuging the hemolymph (3–5 mL) at 180 × *g* for 5 min at 4 °C, the collected hemocytes were resuspended in 1 mL of FASW, layered on a 7 mL Percoll (GE Healthcare) density gradient (10–40%) in FASW, and separated by centrifugation at 500 × *g* for 30 min at 4 °C. The concentrations of the separated hemocytes were individually adjusted to 1×10^5 cells/mL in 1–2 mL FASW. Each hemocyte suspension was immediately mixed with a suspension of fluorescent Alexa Fluor® 488-conjugated *Escherichia coli* bioparticles (ECBP; Invitrogen, Waltham, MA, USA) at a final density of $1–10 \times 10^7$ particles/mL in 10-mL vials. After incubation for 2 h at 4 °C, the hemocytes were washed with 5 mL of FASW, and the fluorescence of extracellular ECBP was quenched by re-suspending the cells in 1 mL of 0.4% Trypan blue solution (Gibco BRL Life Technologies, Grand Island, NY, USA) [26,27], of which salinity was adjusted by adding NaCl to a final concentration of 0.5 M. The hemocytes were then rinsed with FASW, placed on glass slides (S2215, Matsunami), and observed under an Eclipse E600 fluorescence microscope (Nikon) equipped with a fluorescein isothiocyanate (FITC) filter system (Ex, 465–495 nm and Em, 515–555 nm). The percentage of each hemocyte population containing phagocytosed ECBP was determined by counting 200–300 cells in four samples of each of the three mussel species.

2.8. Lysosome detection with LysoTracker®

Hemocyte suspensions (1–2 mL; final concentration of 1×10^5 cells/mL in FASW) were mixed with ECBP suspensions at a final density of $1–10 \times 10^7$ particles/mL in a 10-mL vial, and incubated at 4 °C for 2 and 24 h. The hemocytes were then washed with FASW and stained with 100 nM LysoTracker® Red (LR) solution, which was prepared by diluting LysoTracker® Red DND-99 solution in dimethyl sulfoxide (Molecular Probes, Invitrogen) 10^4 times with FASW. The hemocytes were then lightly rinsed with FASW, and the extracellular ECBPs were quenched by adding 2 mL of 0.4% Trypan blue in FASW. Cells were then washed with FASW and observed using a fluorescence microscope (Optiphot, Nikon). Green and red fluorescence were observed using FITC (Ex, 465–495 nm and Em, 515–555 nm) and CY3 (Ex, 530–570 nm and Em, 573–648 nm) fluorescence filter systems, respectively. The percentages of each hemocyte cell type exhibiting fusion between phagosomes containing ECBP and lysosomes were then determined. For these analyses, 200–300 cells were examined in 4 individual samples of each of the three mussel species.

2.9. Statistical analysis

Statistical analyses were conducted using Microsoft Excel 2008 software (version 12.1.0; Microsoft Corporation, Redmond, WA, USA). The Student's *T*-test was carried out to compare the phagocytic activities (percentage of hemocytes having more than one phagocytosed particle) and the rates of phagosome–lysosome fusion (percentages of hemocytes having one or more orange fluorescent particles [mixture of red fluorescence derived from LR-

stained lysosomes and green fluorescence from ECBP]) of the different hemocyte populations after 2 h and 24 h of incubation. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Classification and characterization of hemocytes

3.1.1. Morphological characterization of hemocytes

Three types of hemocytes were identified by MGG staining of the hemolymph from each of the three mussel species (Fig. 1). Small spherical cells that were 4.8–6.0 μm in diameter, with nuclei that were 3.6–4.3 μm in diameter, were referred to as agranulocytes (AGs; Table 1). The AGs had basophilic cytoplasm with a nucleus/cytoplasm (N/C) diameter ratio of 65–83%, but lacked apparent granules (Fig. 1a, g, and h). While no granule was detected in the AGs by analysis of semi-thin sections granules, vacuole-like structures were occasionally observed (Fig. 1d).

Oval-shaped hemocytes, 6.7–8.0 μm in diameter, which contained basophilic cytoplasm with granules that stained a color similar to that of the cytoplasm, were named basophilic granulocytes (BGs; Fig. 1b, g, and h). While we were unable to clearly observe each of the granules in the whole mounted preparations, the color of the periphery of the granules appeared, to be darker (arrow in Fig. 1b, g, and h). Likewise, in the semi-thin sections, the color of the periphery of each of the granules appeared darker (Fig. 1e). Meanwhile, the nuclei of the BGs were round or ovoid and were 3.6–4.2 μm in diameter, and the N/C ratio was 49–59% (BGs; Table 1).

The third type of hemocyte observed in the hemolymph of the three mussel species was round or ovoid in shape, with a diameter of 8.4–10.0 μm. These cells, referred to as eosinophilic granulocytes (EGs), had oval nuclei, with diameters of 3.6–4.2 μm (EGs; Table 1), and the N/C ratio was 39–48%. The EGs contained eosinophilic cytoplasm with many granules that stained a similar color by MGG-staining (EGs; Fig. 1c, g, and h). While analysis of whole mount preparations indicated that the periphery of each of the granules was noticeably darker in color (arrow in Fig. 1c, g, and h), the periphery of the granules was not darker in the semi-thin section preparations (Fig. 1f).

Similar population densities of total hemocytes were observed in the hemolymph of each of the three *Bathymodiolus* mussel species: $9.0 \pm 1.2 \times 10^5$ cells/mL in *B. japonicus*, $8.4 \pm 2.2 \times 10^5$ cells/mL in *B. platifrons*, and $13.3 \pm 4.4 \times 10^5$ cells/mL in *B. septemdierum* (Table 1). Of the three types of hemocytes, there was an abundance of AGs and EGs in the hemolymph of all three *Bathymodiolus* mussels, accounting for 44.7–48.5% and 34.3–41.0% of the circulating hemocytes in these animals, respectively. Conversely, the BGs were less abundant, accounting for 14.3–17.6% of the total hemocytes (Table 1).

3.1.2. Gravitational densities of hemocytes

Hemocytes from each of the three mussel species were separated into three distinct layers by Percoll density gradient centrifugation (Fig. 2a). Each layer contained one dominant hemocyte type in addition to small amounts of the other hemocyte types (Fig. 2b–d). The uppermost layer, with gravitational densities (gd) of 1.015–1.021 g/mL, was dominated (80.4–91.7%, average = 84.3%) by the AGs (layer I in Fig. 2a, b, and Table 2) in all three *Bathymodiolus* mussel species. Meanwhile, the middle layer (gd = 1.031–1.036 g/mL) was dominated (67.7–84.6%, average = 75.7%) by the BGs (layer II in Fig. 2a, c, and Table 2), and the heaviest layer (gd = 1.038–1.048 g/mL) was dominated (89.9–93.3%, average = 91.2%) by the EGs (layer III in Fig. 2a, d, and Table 2).

3.1.3. Transmission electron microscopy (TEM) of hemocytes

TEM analysis revealed that the AGs contained no granules; however, short thread-like pseudopodia were occasionally

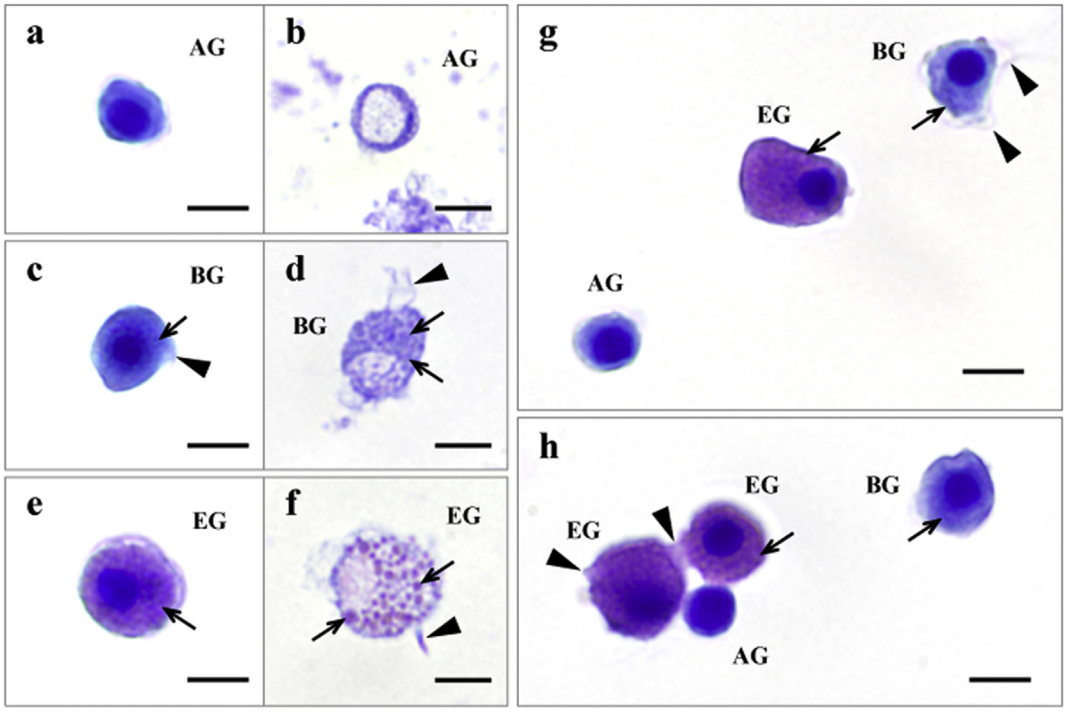

Fig. 1. Light micrographs of the hemocytes of the three *Bathymodiolus* mussel species stained with May-Grünwald–Giemsa (MGG) stain. a–c: Whole mounted hemocytes of *B. japonicus*; d–f: Semi-thin sections (thickness, 1 μm) of the hemocytes of *B. japonicus*. MGG staining of the semi-thin section resulted in strong staining of the granules but relatively faint staining of the nuclei. g: Whole mounted hemocytes of *B. platifrons*; and h: Whole mounted hemocytes of *B. septemdierum*. AG, agranulocyte; BG, basophilic granulocyte; EG, eosinophilic granulocyte. Arrows, granules; arrowheads, pseudopodia; scale bar, 5 μm.

Table 1
Cell sizes and populations of hemocytes in three *Bathymodiolus* mussels.

Mussel species	Symbiotic bacteria	Total hemocyte population density[c,e]	Cell type	Percentage of hemocyte population[f] (%)	Cell diameter[g] (μm)	Nuclear diameter[g] (μm)	N/C ratio[d,g] (%)
B. japonicus[a]	Methane-oxidizing bacteria	9.0 ± 1.2	AG	44.7 ± 7.9	5.4 ± 0.6	4.0 ± 0.3	74 ± 9
			BG	14.3 ± 4.3	7.6 ± 0.4	4.0 ± 0.2	53 ± 4
			EG	41.0 ± 9.1	9.2 ± 0.8	3.9 ± 0.1	43 ± 4
B. platifrons[a]	Methane-oxidizing bacteria	8.4 ± 2.2	AG	45.8 ± 3.8	5.5 ± 0.3	4.1 ± 0.2	75 ± 5
			BG	17.6 ± 5.5	7.3 ± 0.5	3.9 ± 0.3	55 ± 5
			EG	36.6 ± 2.5	9.2 ± 0.7	4.0 ± 0.2	44 ± 4
B. septemdierum[b]	Sulfur-oxidizing bacteria	13.3 ± 4.4	AG	48.5 ± 5.2	5.4 ± 0.5	3.8 ± 0.2	71 ± 5
			BG	17.2 ± 4.8	7.2 ± 0.5	3.9 ± 0.2	54 ± 5
			EG	34.3 ± 6.0	9.0 ± 0.6	3.8 ± 0.2	43 ± 4

AG, agranulocyte; BG, basophilic granulocyte; EG, eosinophilic granulocyte.
[a] From a seep off Hatsushima, Sagami-bay, Japan.
[b] From a hydrothermal vent at Myojin Knoll, Ogasawara, Japan.
[c] $\times 10^5$ cells/mL hemolymph.
[d] Nucleus/cytoplasm diameter ratio.
[e] Means of 4 individuals.
[f] Means of 7 individuals.
[g] Means of 200 cells.

observed (Fig. 3a). Considerable numbers of mitochondria and a conspicuous amount of rough and smooth endoplasmic reticulum were also observed (Fig. 3d). In addition, the Golgi apparatus and small vacuoles were often observed in the cytoplasm of these cells (Fig. 3d).

The BGs generally had several prominent, elongated pseudopodia (arrowheads in Fig. 1b, e, and g; labeled as ep in Fig. 3b), and a few phagosome-like vacuoles were observed in the peripheral region of the cytoplasm of these cells (arrow in Fig. 3b). In addition to several organelles, such as mitochondria, endoplasmic reticulum, and Golgi apparatus (Fig. 3e), the cytoplasm of the BGs contained conspicuous spherical granules (0.3–0.4 μm in diameter; arrowheads in Fig. 3e), which were likely the basophilic granules observed by light microscopy (Fig. 1b, e). The electron density of

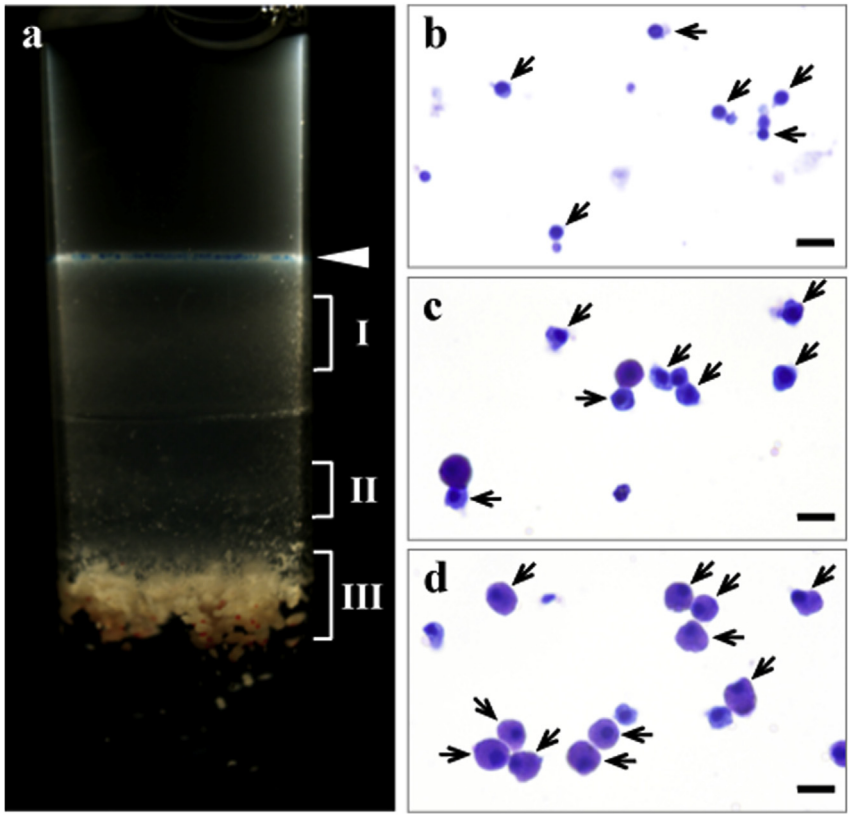

Fig. 2. Percoll density gradient centrifugation of glutaraldehyde-fixed hemocytes in *B. japonicus*. Hemocytes were stained with May-Grünwald–Giemsa (MGG) stain. a: Hemocytes of *B. japonicus* were separated into three distinct layers by Percoll density gradient centrifugation. Layer I, uppermost layer; II, middle layer; III, heaviest layer. Arrowhead, standard density marker bead (1.013 g/mL). b–d: Light micrographs of the separated hemocytes after staining with MGG. b: Layer I was dominated by agranulocytes (arrows); c: Layer II was dominated by basophilic granulocytes (arrows); d: Layer III was dominated by eosinophilic granulocytes (arrows). Scale bar, 10 μm. In c and d, while the basophilic or eosinophilic granules were not evident at this low magnification, BGs and EGs were recognizable by their respective basophilic (blue) and eosinophilic (pink) cytoplasm.

Table 2
Percoll density gradient separation of glutaraldehyde-fixed hemocytes in *Bathymodiolus* mussels.

Mussel species	*B. japonicus*			*B. platifrons*			*B. septemdierum*		
Density gradient layers	Uppermost layer	Middle layer	Heaviest layer	Uppermost layer	Middle layer	Heaviest layer	Uppermost layer	Middle layer	Heaviest layer
Gravitational density[a] (g/mL)	1.017 ± 0.002	1.030 ± 0.002	1.038 ± 0.001	1.021 ± 0.001	1.031 ± 0.003	1.047 ± 0.002	1.015 ± 0.001	1.036 ± 0.004	1.048 ± 0.002
AG (%)[b]	82.8 ± 2.4	20.4 ± 7.2	nd	82.0 ± 0.6	17.7 ± 7.2	nd	88.0 ± 3.7	10.9 ± 1.8	nd
BG (%)[b]	17.2 ± 2.4	73.5 ± 5.8	8.9 ± 0.7	18.0 ± 0.6	73.7 ± 5.5	9.2 ± 0.9	12.0 ± 3.7	80.0 ± 4.6	8.3 ± 1.7
EG (%)[b]	nd	6.1 ± 1.4	91.1 ± 0.7	nd	8.6 ± 3.8	90.8 ± 0.9	nd	9.1 ± 2.8	91. 7 ± 1.7

nd, Not detected.
AG, agranulocyte; BG, basophilic granulocyte; EG, eosinophilic granulocyte.
[a] Means of 4 individuals.
[b] Means of hemocytes from 4 individuals.

these granules was variable, ranging from relatively lucent to more dense. Furthermore, the granules were surrounded by single, limiting membranes, and the granule cores were denser than their peripheries (arrows in Fig. 3e).

A notable characteristic of the EGs was the presence of many large, electron-dense granules in the cytoplasm that were 0.8–1.2 μm in diameter (arrowheads in Fig. 3f), which likely corresponded to the eosinophilic granules observed by light microscopy (Fig. 1c, f). Each granule was surrounded by a single limiting membrane, which contained an electron-dense core (0.6–0.7 μm in diameter; white arrow in Fig. 3f) and an electron-lucent peripheral layer (approximately 60 nm in thickness; black arrow in Fig. 3f). There was some variation in the electron densities of these granules (Fig. 3c and f), and their morphological characteristics were similar to those of the BG granules. However, the granules of the EGs were significantly larger than those of the BGs, and sometimes measured greater than 2.0 μm in diameter (arrowhead in Fig. 3c, Table 1). In addition to the granules, the cytoplasm of these cells also contained organelles such as mitochondria, endoplasmic reticulum, Golgi apparatus (Fig. 3f), and, frequently, phagosome-like vacuoles (data not shown). The EGs also exhibited broad pseudopodia (Fig. 3c).

3.1.4. Lectin binding analyses

Previous studies have shown that lectins can be utilized for the classification and characterization of hemocyte types [10,14,19,21]. The sugar binding specificities of the lectins used as well as the lectin binding characteristics of the three types of hemocytes are summarized in Table 3. WGA bound to both BGs and EGs, but showed no affinity for AGs (Fig. 4a, d, and g). Specifically, this lectin bound to the granules (Fig. 4d and g) and cell membranes (Fig. 4d and g) of the EGs and BGs of each of the mussel species tested (Table 3). Meanwhile, Con-A bound to the cytoplasm and nuclear membrane of all three types of hemocytes (Fig. 4b, e, h, and Table 3) and, like WGA, bound to the granules of both the EGs and BGs (Fig. 4e and h). SBA bound only to the granules in the EGs of *B. japonicus* (Fig. 4i), but not to those in the other hemocyte types in

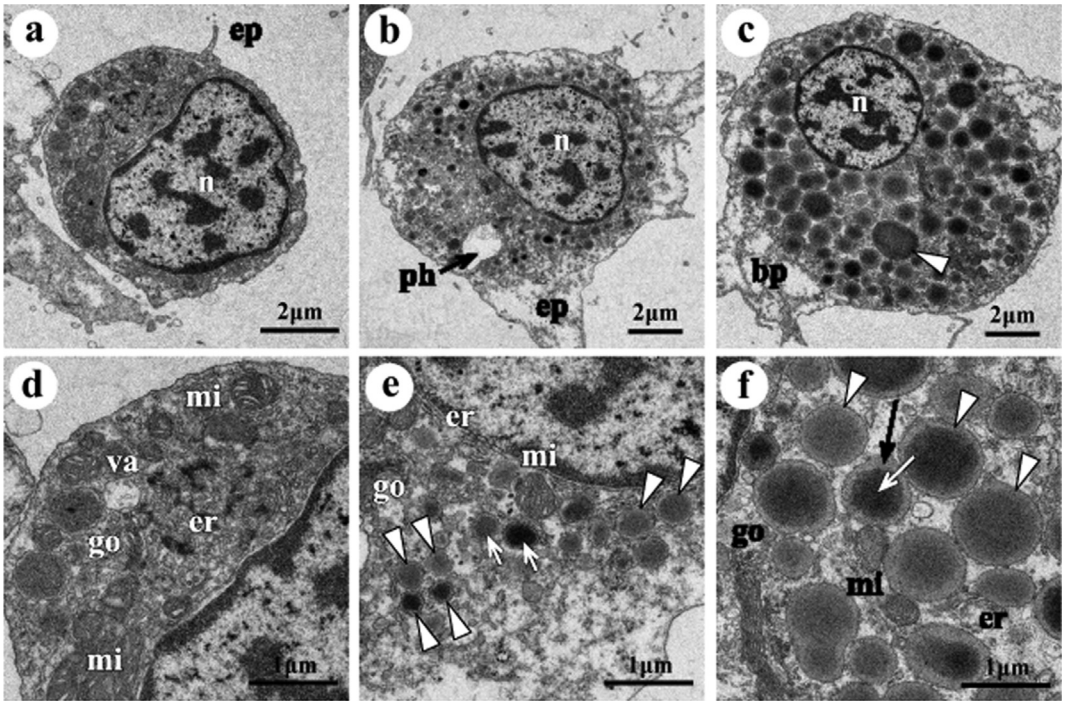

Fig. 3. Transmission electron micrographs of the hemocytes of *Bathymodiolus japonicus*. a and d: Agranulocytes; b and e: Basophilic granulocytes, which contain small electron-lucent to electron-dense granules (arrowheads) with electron dense cores (arrows); c and f: Eosinophilic granulocytes containing large electron-dense granules (arrowhead) with electron-dense cores (white arrow) and more electron-lucent peripheries (black arrow). bp, broad pseudopodia; ep, elongated pseudopodia; er, endoplasmic reticulum; go, Golgi apparatus; mi, mitochondria; n, nucleus; ph, phagosome-like vacuole; va, vacuole.

Table 3
The sugar-binding specificity and hemocyte-binding characteristics of lectins.

Name of lectin (abbreviation)	Lectin source	Sugar-binding specificity	Hemocytes		
			AG	BG	EG
Concanavalin A (Con-A)	*Canavalia ensiformis*	α-D-Mannose, α-D-glucose	Cell membrane	Cell membrane, Granules	Cell membrane, Granules
Phytohemagglutinin-L (PHA-L)	*Phaseolus vulgaris*	D-N-acetylgalactosamine, oligosaccharide	No binding	No binding	No binding
Soybean agglutinin (SBA)	*Glycine max*	D-N-acetylgalactosamine	No binding	No binding	Granules in only *B. japonicus*
Wheat germ agglutinin (WGA)	*Triticum vulgaris*	D-N-acetylglucosamine, sialic acid	No binding	Cell membrane, Granules	Cell membrane, Granules

AG, agranulocyte; BG, basophilic granulocyte; EG, eosinophilic granulocyte.

this species (Fig. 4c, f, and Table 3). Moreover, this lectin failed to bind to the hemocytes of the other two *Bathymodiolus* mussel species tested (Table 3). Lastly, none of the hemocytes of the mussel species tested bound to PHA-L (Table 3).

3.2. In vitro phagocytic activity and phagosome–lysosome fusion

After 2 h of incubation with ECBPs at 4 °C, no phagocytic activity was detected in the AGs (Fig. 5a and d). In contrast, green fluorescent particles were observed in the BGs (Fig. 5b and e) and EGs (Fig. 5c and f) in all three of the mussel species tested, indicating that these cells engulfed the ECBPs. Although the particles were often clumped and could not be counted, more particles seemed to be engulfed by the EGs than by the BGs (Fig. 5e and f).

Fusion of phagosomes with LR-stained lysosomes was also examined in each of the three mussel species. After incubating the hemocytes with the ECBPs for 2 and 24 h, and then with LR for an additional 1 h, lysosomes were examined by fluorescence microscopy. The ECBPs in phagosomes that did not fuse with lysosomes exhibited green fluorescence. In contrast, after fusion, phagolysosomes exhibited orange fluorescence, which resulted from the mixing of the green ECBP fluorescence and the red fluorescence of the LR during the acidification of the phagosome. Neither fluorescent particles nor LR-stainable lysosomes were detected in the AGs (Fig. 6a and d) of any of the three mussel species, even after 24 h of incubation, indicating that this type of hemocyte was both non-phagocytic and lacked lysosomes. Conversely, fluorescent particles were detected in both the BGs and EGs (Fig. 6b and c) of each of the three mussel species tested. While most of the ECBPs ingested by the EGs exhibited orange fluorescence after 2 h of incubation, indicating phagosome–lysosome fusion, some of the particles remained green (Fig. 6c). Meanwhile, fewer ECBPs were engulfed by the BGs, and most retained their green fluorescence (arrowhead in Fig. 6b), indicating that phagosome–lysosome fusion did not occur within 2 h.

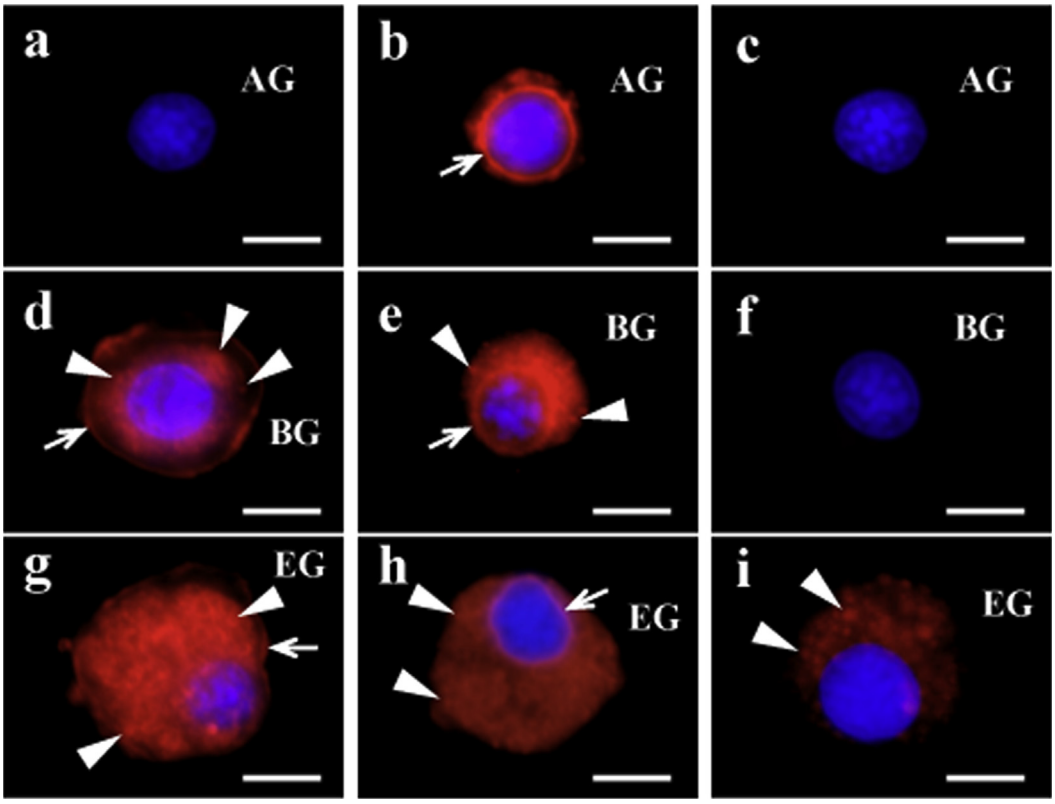

Fig. 4. Fluorescence micrographs of hemocytes harvested from *Bathymodiolus japonicus* treated with fluorophore-conjugated lectins. a–c: Agranulocyte (AG); d–f: Basophilic granulocyte (BG); and g–i: Eosinophilic granulocyte (EG). Wheat germ agglutinin (WGA; a, d, and g) failed to bind AGs (a) but bound to the cell membranes (d and g; arrow) and granules (d and g; arrowheads) of both BGs (d) and EGs (g). Concanavalin agglutinin (Con-A; b, e, and h) bound to the perinuclear membranes (b, e, and h; arrow) and granules (e and h; arrowheads) of all three types of hemocytes (b, e, and h). Soybean agglutinin (SBA; c, f, and i) bound only to the granules in the EGs of *B. japonicus* (i; arrowheads), but not to the other hemocyte types (c and f). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Scale bar, 5 μm.

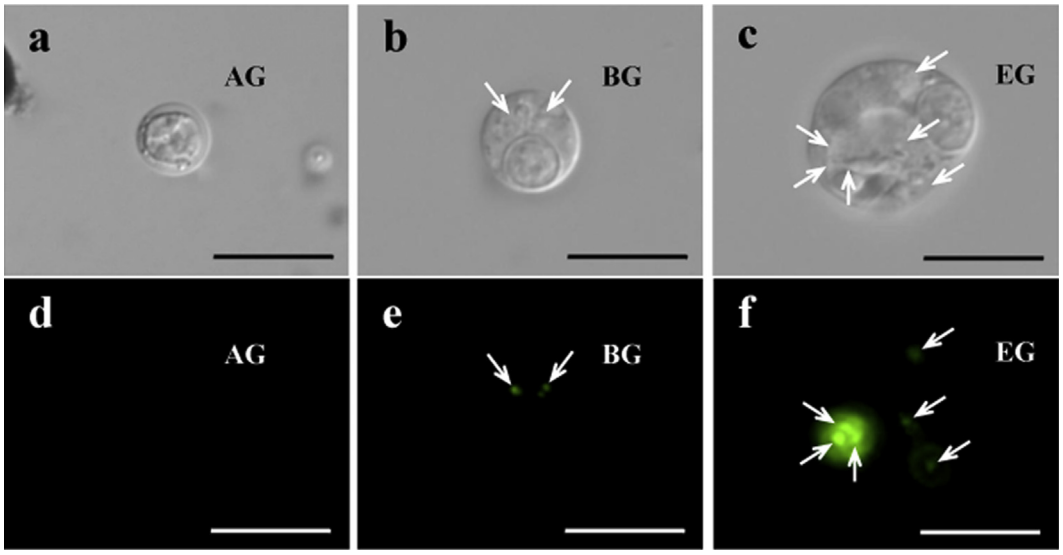

Fig. 5. Phagocytosis by the hemocytes of *Bathymodiolus japonicus*. Phagocytosis was observed by differential interference contrast microscopy (a, b, and c) and fluorescence microscopy (d, e, and f). a and d: Agranulocyte (AG). Phagocytosis of the *Escherichia coli* bioparticle (ECBP) was not observed. b and e: Basophilic granulocyte (BG); and c and f: Eosinophilic granulocyte (EG). Arrows in b, c, e, and f indicate phagocytosed ECBPs. Scale bar, 10 μm.

After incubation for 24 h, the BGs of *B. japonicus* and *B. platifrons* engulfed greater numbers of ECBPs (arrow in Fig. 6e). Indeed, the phagosomes of these cells exhibited red fluorescence and contained more particles than did those of the BGs after 2 h of incubation. Furthermore, toward the end of the incubation time, the majority of the engulfed particles exhibited orange fluorescence (arrow in Fig. 6e). However, the fluorescence of certain phagocytosed ECBPs remained green, indicating that some phagosomes were not acidified, even after 24 h of incubation (data not shown). Likewise, the EGs also engulfed more ECBPs, and the phagosomes became larger after 24 h (Fig. 6f). The majority of the particles engulfed by these cells exhibited orange fluorescence (arrows in Fig. 6f), although some remained green (arrowhead in Fig. 6f).

The percentages of hemocytes that phagocytosed one or more ECBPs, as well as the percentages of those cells that exhibited phagosome–lysosome fusion (indicated by orange fluorescence of the ingested particles) are depicted in Fig. 7. None of the AGs exhibited phagocytosis, even after 24 h of incubation (Fig. 7a). After 2 h of incubation, the percentage of EGs that ingested ECBPs (68.4–76.3% of the total EG population) was significantly higher than the percentage of BGs that ingested ECBPs (12.7–16.2%) in all three species tested (Fig. 7a). After incubation for 24 h, the

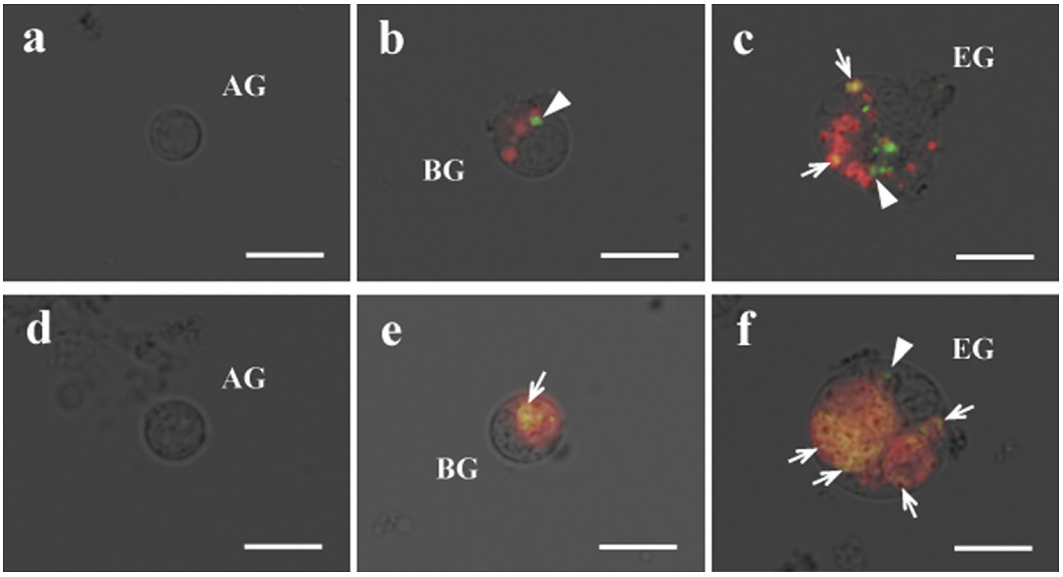

Fig. 6. Analysis of phagosome–lysosome fusion in the hemocytes of *Bathymodiolus japonicus*. *B. japonicus* hemocytes were incubated with *Escherichia coli* bio-particles (ECBP) for 2 h (a, b, and c) or 24 h (d, e, and f), and then with LysoTracker® Red solution for an additional 1 h, and observed under a fluorescence microscope. a and d: Agranulocyte (AG); b and e: Basophilic granulocyte (BG); and c and f: Eosinophilic granulocyte (EG). Arrowheads indicate green fluorescent ECBPs present in the phagosome prior to lysosome fusion. Arrows indicate orange fluorescent ECBPs present in phagolysosomes. Scale bar, 10 μm.

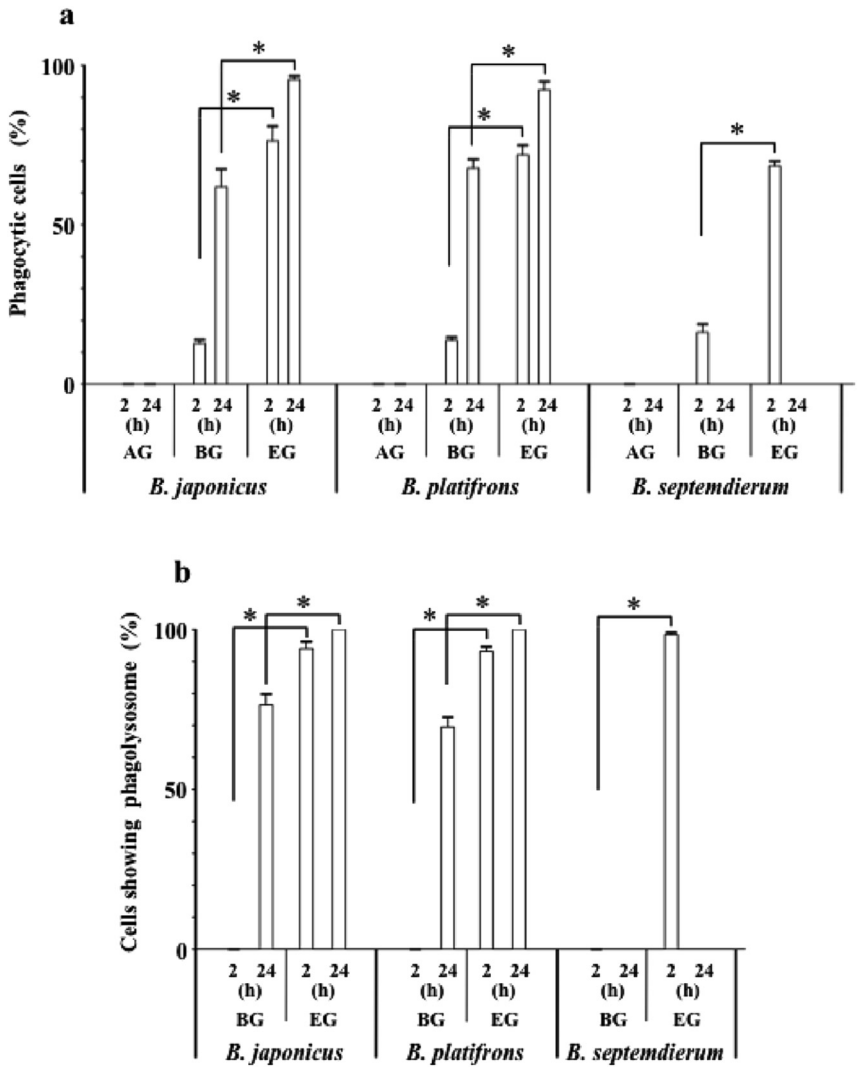

Fig. 7. Percentages of phagocytic *Bathymodiolus* hemocytes, and of hemocytes containing phagolysosomes. a: The percentages of phagocytic cells that engulfed one or more ECBPs; and b: The percentages of phagocytic cells containing one or more orange-fluorescent ECBP, indicating phagosome–lysosome fusion. AG, agranulocyte; BG, basophilic granulocyte; EG, eosinophilic granulocyte. *Indicates statistically significant ($P < 0.05$) differences between the percentages of BGs and EGs in each *Bathymodiolus* species exhibiting phagocytosis (a) and phagosome–lysosome fusion (b), respectively. Bar, mean ± standard deviation (SD; $n = 4$) of the results of four independent experiments. *B. septemdierum* was not available for the 24-h incubation experiment.

percentages of BGs (61.9–67.7%) and EGs (92.3–95.5%) that engulfed particles increased in both *B. japonicus* and *B. platifrons* (Fig. 7a). However, the differences between these two hemocyte populations remained significant, even after 24 h of incubation (Fig. 7a). Phagosome–lysosome fusion was not observed after 2 h of incubation in the BGs of any of the three mussel species tested (Fig. 7b). However, lysosomal fusion increased to 69.5–76.4% after 24 h of incubation (Fig. 7b). Meanwhile, the percentages of EGs containing phagolysosomes were 93.2–98.4% after 2 h of incubation (Fig. 7b) and 100% after 24 h of incubation (Fig. 7b), which were significantly greater than the percentages observed in the BGs (Fig. 7b). Unfortunately, *B. septemdierum* mussels could not be obtained for the 24-h incubation experiment and were therefore not tested.

4. Discussion

The results of this study revealed that, despite differences in their habitats and symbiotic bacteria, the three symbiotic *Bathymodiolus* mussel species examined each possess three distinct types of hemocytes: AGs, BGs, and EGs (Supplementary Table S1). Photosymbiotic clams belonging to the family Cardiidae have been reported to have unique hemocytes, referred to as morula-like cells, which were not detected in closely related non-symbiotic Cardiidae clams [28]. The *Bathymodiolus* mussels, however, seem to have no hemocyte population that is specific to the symbiotic deep-sea mussels (Table S1).

In most mytilid mussels, while the AGs have similar morphological characteristics, they have been referred to by different names: hemoblast-like cells in *B. azoricus* [19], basophilic agranulocytes in *M. edulis* [14,29], and hyalinocytes in *M. galloprovincialis* [16]. While these cell types have been reported to show weak phagocytic activity, the AGs in the present study were non-phagocytic (Supplementary Table S1, Fig. 7). In a previous study, Hine reported that the AGs in bivalves can be grouped into two categories: (i) blast-like cells with a central ovoid or spherical nuclei surrounded by a thin cytoplasm lacking organelles, and (ii) AGs (or hyalinocytes) with larger ovoid, reniform or irregular eccentric nuclei, and cytoplasm containing a variety of organelles [11]. Comparisons of the morphological features of the nuclei and organelles suggested that the AGs of the *Bathymodiolus* mussels examined in this study belong to the latter group (Fig. 3a and d).

The hemoblast or blast cell is thought to be an undifferentiated hemocyte that differentiates into granulocytes [11,30,31]. Cell division or DNA synthesis in the blast cell or small sized cell, has been reported in the Manila clam [31] and in the snail *Littorina littorea* [32]. In abalone, however, the DNA contents of the blast-like cells were found to be the same as those of the other types of hemocytes, indicating that these cells do not proliferate within the hemolymph [33]. On the contrary, it was suggested that granulocytes differentiate into hyalinocytes via degranulation [13]. Although it is difficult to rule out the possibility that the AGs in the *Bathymodiolus* mussels are actually blast cells, which differentiate into other types of hemocytes, with or without proliferation, no intermediate type of hemocyte (between the AGs and the granulocytes [EGs and BGs]) was detected in any of the three *Bathymodiolus* mussels examined in the present study.

While the percentages of AGs among the hemocytes of all three *Bathymodiolus* mussel species examined in this study (Table 1) were markedly higher than the prevalence of hemoblast-like cells or agranular type hemocytes in *B. azoricus*, *M. edulis*, and *M. galloprovincialis* [19,29,34] (Supplementary Table S1), the specific function of these AGs is unknown. The agranular amebocytes found in the pearl oyster *Pinctada fucata* are known to produce an extracellular matrix that aids in wound healing [35,36]. However, further studies are needed to understand the role of AGs in immunity and/or wound healing.

The characteristics of the EGs of the three *Bathymodiolus* mussels were similar to those of the granulocytes in *B. azoricus* [19], the eosinophilic granulocytes or granular eosinophils in *M. edulis* [14,29], and to the granulocytes or acidophilic granulocytes in *M. galloprovincialis* (Supplementary Table S1) [16,18]. Although these cells differ slightly from each other, they could all be classified as EGs. These cells are likely professional phagocytes, which eliminate foreign microorganisms that invade the hemolymph and other tissues.

The characteristics of the BGs of the three *Bathymodiolus* species were similar to those of the hyalinocytes in *B. azoricus* [19], the basophilic granulocytes or granular basophils in *M. edulis* [14,29], and the basophilic granulocytes in *M. galloprovincialis* [16,23,24] (Supplementary Table S1). These similarities indicate that each of these cell types could be classified as BGs. An intermediate type of granulocyte containing both acidophilic and basophilic granules, which may comprise a transitional cell population between the two types of granulocytes, was reported in *M. galloprovincialis* [16]. However, this type of hemocyte was not observed in any of the three *Bathymodiolus* mussel species analyzed in this study.

In the lectin-binding assays performed in this study, WGA bound specifically to the EGs and BGs, but not to the AGs (Table 3), while SBA bound only to the granules in the EGs of *B. japonicus* (Fig. 4c, f, and i). These findings suggest that lectins can be useful for differentiating hemocyte types, and that the BGs and EGs of *B. japonicus*, and perhaps other mussel species, differ, not only with respect to their granular size and MGG stainability, but also in their sugar moieties. As shown in *B. azoricus*, WGA binding of sugar moieties may be involved in granulocyte–granulocyte contact and in phagocytic recognition [19]. Meanwhile, in *M. edulis*, the smaller and larger granules, which are contained in two distinct types of granulocytes, have been shown to specifically bind HPA (*Helix pomatia* agglutinin) and WGA, respectively [20]. Furthermore, these two types of granulocytes can be distinguished using monoclonal antibodies [37]. These data suggest that the BGs and EGs of the *Bathymodiolus* mussels comprise distinct hemocytes populations, which may express different sugar moieties and perform disparate functions.

The granules of the BGs and EGs were similar to each other in ultrastructure. Furthermore, these granules are similar to those present in the granulocytes of *M. galloprovincialis*, which have been suggested to function as lysosomes [18]. Indeed, lysosomal enzymes have been detected in the granules of the granular hemocytes of *M. edulis* [38]. In this study, the granules that stained LR-positive in the BG and EG populations are thought to be lysosomes (Fig. 6). However, the acidophilic/basophilic nature of these granules suggests differences in their enzymatic contents, which is consistent with a previously proposed hypothesis [14,38–40].

Among the hemocyte populations, the EGs exhibited the highest level of phagocytic activity (Fig. 7a). It was previously reported that the eosinophilic granular hemocytes exhibit higher phagocytic activity than the basophilic granular hemocytes in various mytilid mussel species [11,14,19,23]. In addition to phagocytosis, the percentage of EGs that exhibited phagosome–lysosome fusion was significantly higher than that of BGs (Fig. 7b). However, it is unclear whether this can be attributed to slower initiation of fusion between the phagosome and lysosome after phagocytosis, or if this simply reflects the low phagocytic activity of BGs. Regardless, the results of the present study suggest that the EGs have higher intracellular digestive activity than the BGs. Previous studies demonstrated that the eosinophilic granular hemocytes of *M. edulis* exhibit high levels of phagocytic activity and contain various lysosomal enzymes, including phenol oxidase, peroxidase, and arylsulphatase [14,38]. Meanwhile, the basophilic granulocytes of *M. edulis* and *M. galloprovincialis* were shown to have lower phagocytic activity and fewer lysosomal enzymes than the eosinophilic granular hemocytes [14,18,23,38]. The lysosomal content of the granulocytes (corresponding to the EGs in the present study) was reported to be greater than that of the hyalinocytes (the BGs in the present study) in *Perna viridis* [41], *Mya arenaria* [42], *Haliotis discus discus*, and *Turbo cornutus* [43]. Moreover, the differences in the gravitational densities between the BGs and EGs (Table 2) may reflect differences between these two cell types in the amounts of granules (i.e., lysosomes) they possess. Although both the BGs and EGs exhibited phagocytic activity, the observed differences between the activities of these cells may indicate that they play distinct roles in the host immune system. For example, they may target different types of microorganisms. There is precedence for this, as the granulocytes of *Crassostrea gigas* were found to exhibit higher levels of phagocytic activity against bacteria and yeast than the agranulocytes, while the agranulocytes exhibited higher levels of phagocytosis against human erythrocytes and latex beads than the granulocytes [44]. The phagocytic activity and post-phagocytosis lysosomal maturation processes may be divergent in different types of hemocytes, and their functional roles may be different among various bivalves living in distinct environments. Comparative studies are therefore necessary to address these questions.

In *M. galloprovincialis*, the three types of hemocytes (hyalinocytes, small granulocytes, and large granulocytes) were shown to exhibit distinct responses to infection by different bacterial species such as *Vibrio* and *Micrococcus* [45]. In a study of insect–symbiont interactions, it was reported that host *Aphid* hemocytes (granulocytes) engulf the secondary symbionts and, to a lesser extent, the primary symbionts [46]. Meanwhile, the way in which the hemocytes of *Bathymodiolus* mussels, particularly the BGs and EGs, interact with the symbiotic bacteria is a topic of great interest. In juvenile *Bathymodiolus* mussels, it was recently shown that symbiotic bacteria reside, not only in the gill tissue, but also within the epithelial cells of the mantle and foot, and that these bacteria disappear from tissues other than the gill during the later ontogeny [47]. It would be interesting to determine whether hemocytes are involved in the elimination of the symbiotic bacteria during early development.

In conclusion, the *Bathymodiolus* mussel species examined in this study were found to contain three distinct types of hemocytes:

AGs, BGs, and EGs. The characteristic features of these hemocytes were similar to those of corresponding hemocytes in the mussels belonging to the family Mytilidae. While the AGs exhibited no phagocytic activity, the BGs and EGs were phagocytic and displayed post-phagocytic phagosome–lysosome fusion; however, the levels of these activities were different between the two granular hemocyte populations. Further studies are required to elucidate the roles of these different hemocyte types in the host immune system.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2015.03.020.

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